High CO_2 chemosensitivity *versus* wide sensing spectrum: a paradoxical problem and its solutions in cultured brainstem neurons

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CO_2 central chemoreceptors play an important role in cardiorespiratory control. They are highly sensitive to P_{CO_2} in a broad range. These two sensing properties seem paradoxical as none of the known pH-sensing molecules can achieve both. Here we show that cultured neuronal networks are likely to solve the sensitivity *versus* spectrum problem with parallel and serial processes. Studies were performed on dissociated brainstem neurons cultured on microelectrode arrays. Recordings started after a 3 week initial period of culture. A group of neurons were dose-dependently stimulated by elevated CO_2 with a linear response ranging from 20 to 70 Torr. The firing rate of some neurons increased by up to 30% in response to a 1 Torr P_{CO_2} change, indicating that cultured brainstem neuronal networks retain high CO_2 sensitivity in a broad range. Inhibition of Kir channels selectively suppressed neuronal responses to hypocapnia and mild hypercapnia. Blockade of TASK channels affected neuronal response to more severe hypercapnia. These were consistent with the pK_a values measured for these K^+ channels in a heterologous expression system. The CO_2 chemosensitivity was reduced but not eliminated by blockade of presynaptic input from serotonin, substance P or glutamate neurons, indicating that both pre and postsynaptic neurons contribute to the CO_2 chemosensitivity. These results therefore strongly suggest that the physiological P_{CO_2} range appears to be covered by multiple sensing molecules, and that the high sensitivity may be achieved by cellular mechanisms via synaptic amplification in cultured brainstem neurons.

Spontaneous breathing requires continuous feedback controls by respiratory gases. Whereas O_2 detection is carried out by peripheral chemoreceptors, CO_2 sensing mainly depends on the central chemoreceptors (CCRs) known to be located in several brainstem areas (Feldman *et al.* 2003; Putnam *et al.* 2004; Richerson, 2004; Guyenet *et al.* 2005*b*). A mystery about the CCRs is how brainstem neurons manage to detect P_{CO_2} as low as 1 mmHg, couple it to a 20–30% change in ventilation, and meanwhile cover a broad range of P_{CO_2} (Nattie, 1999; Putnam *et al.* 2004). Sensitivity is an inherent property of CO_2/pH sensing molecules that is determined by the steepness of the pH-response curve. A steep response, however, confines the sensing molecule to a narrow pH range. In contrast, a sensing molecule that covers a wide spectrum of P_{CO_2} tends to have very low sensitivity. Indeed, none of the known CO_2/pH-sensing molecules is capable of producing

a change in membrane potentials or cellular activity by 20–30% in response to 1 mmHg P_{CO_2} (Jiang *et al.* 2005). Exactly how the respiratory neuronal networks solve these seemingly paradoxical problems is unknown.

Several groups of brainstem neurons are CO_2 chemosensitive, such as serotonergic neurons in the midline raphe nuclei, glutamatergic neurons in the retrotrapezoid nucleus, and catecholaminergic neurons in the locus coeruleus (Pineda & Aghajanian, 1997; Oyamada *et al.* 1998, 1999; Wang *et al.* 1998; Stunden *et al.* 2001; Filosa & Putnam, 2003; Severson *et al.* 2003; Mulkey *et al.* 2004; Ritucci *et al.* 2005). These neurons are not respiratory modulated, but they project to respiratory-modulated neurons, indicating that these CO_2 chemosensitive cells are presynaptic (Richerson, 2004; Guyenet *et al.* 2005*b*). Brainstem respiratory-modulated neurons are also CO_2 chemosensitive. In response to hypercapnia, inspiratory neurons are depolarized, and expiratory cells are hyperpolarized. These neuronal responses are retained after blockade of synaptic transmission, indicating that the

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postsynaptic cells are intrinsically CO_2 chemosensitive (Onimaru *et al.* 1989; Kawai *et al.* 1996; Okada *et al.* 2002; Guyenet *et al.* 2005a; Kawai *et al.* 2006). The presence of CO_2 chemosensitivity in both pre and postsynaptic neurons suggests a potential amplification mechanism by these neurons. Since these neurons are arranged in series in their network, we designated the neuronal process of CO_2 signals as the serial process.

Several ion channels and receptors are modulated by physiological levels of P_{CO_2}/pH, and may act as sensors, including several Kir channels, TASK1 channel, Ca^{2+} channels, TEA-sensitive K^+ channels, gap junctions, P2x receptor, non-selective cationic channels, etc. (Dean *et al.* 1997; Thomas *et al.* 1999; Talley *et al.* 2000; Richerson *et al.* 2001; Putnam *et al.* 2004; Jiang *et al.* 2005). These potential CO_2/pH sensing molecules coexist in several brainstem nuclei (Talley *et al.* 2000; Bradley *et al.* 2002; Washburn *et al.* 2002; Wu *et al.* 2004). Such a wide existence of these molecules suggests a process by which CO_2 signals are detected by multiple sensing molecules working in parallel. We thereby named the latter neuronal process of CO_2 signals the parallel process.

To find evidence for the serial and parallel processes, we performed studies in cultured brainstem neurons on microelectrode arrays (MEAs). The MEA technology allows recordings of neuronal activity in regular culture medium without evident perturbation of intra and extracellular environment such as temperature, pH, osmolarity, ionic composition, second messengers, etc. More importantly, the understanding of pre and postsynaptic processes of P_{CO_2} signal requires simultaneous recordings from multiple neurons in their networks and *de novo* dissection of the synaptic mechanisms; the MEA is ideal for both in a reduced neuronal network (Su & Jiang, 2006).

Methods

Cell culture in microelectrode arrays

MEA dishes were purchased from ALA Scientific (Westbury, NY, USA), a distributor of MCS (Reutlingen, Germany), with each dish having 64 microelectrodes for which the tip diameter is 30 μm and the interelectrode space is 200 μm.

The preparation of primary neuronal culture was the same as we previously described (Su & Jiang, 2006). In brief, a timed-pregnant embryonic Sprague-Dawley rat (17–19 days) was anaesthetized with inhalation of saturated halothane (Halocarbon Laboratories, River Edge, NJ, USA), according to IACUC-approved protocols for the care and use of laboratory animals. Embryos were removed and chilled on ice. Under sterile conditions, all tissues from the lower brainstem containing the whole medulla and pons were cut into tissue blocks (0.5–1 mm^3). From each fetus, 4–5 brainstem tissue pieces of $\sim$0.5 mm

thickness were obtained, and each piece was split into two. The tissue pieces from all fetuses in one litter were mixed together and digested in a solution containing papain (Worthington-Biochem, Lakewood, NJ, USA) for 30 min in an incubator with 5% CO_2 and 95% air, at 37°C. The digested tissue pieces were triturated by using a P-1000 Pipetman. Passing through a 40 μm Falcon filter (BD Biosciences, Bedford, MA, USA), the solution was centrifuged at 300 *g* for 5 min. After discontinuous density gradient centrifugation with albumin-inhibitor solution at 70 *g* for 6 min, the cell pellet was immediately resuspended in culture medium according to the protocol supplied by the manufacture (Worthington-Biochem). Dissociated cells (20 000–50 000) in a 20 μl droplet were plated onto the 1.5 mm^2 electrode region of an MEA dish. With this protocol, each pregnant animal can be used for 8–10 MEA dishes. The dish was then filled with 1.2 ml neurobasal medium (NBM) supplemented with B27 and GlutaMaxI. The solution was changed to Dulbecco's modified Eagle's medium (DMEM) containing 10% horse serum (Gibco/Invitrogen, Carlsbad, CA, USA) after 2 days. To reduce the variability among MEA dishes, great efforts were made to keep the cell mixture as homogeneous as possible, and recordings were performed under the same experimental conditions (Su & Jiang, 2006). However, there was certain variability among MEA dishes, and some dishes showed more responsive neurons than others; this is likely to be caused by the proportion of survival neurons from certain brainstem areas in each dish.

The MEA dish was covered with a Teflon MEA lid and tightly sealed with fluorinated ethylene-propylene membrane (Teflon FEP film, American Durafilm, Holliston, MA, USA). This film is permeable to CO_2/O_2, but not to microbes and water vapour (Potter & DeMarse, 2001). Cells were cultured at 36°C, with 5% CO_2 and 95% air in a cell culture incubator (Model NU-4750; Nuaire, Plymouth, MN, USA).

MEA recording

Extracellular recordings were carried out at 36°C in DMEM by using a preamplifier (MEA1060-2; MCS) that held one MEA dish and was kept in an incubator during recording. Spikes were digitized at 40 kHz with a 64-channel A/D converter and the MEA Workstation software (Plexon, Dallas, TX, USA). Single-unit activity was then identified using the OfflineSorter software (Plexon) based on principal component analysis methods (Horn & Friedman, 2003). CO_2 exposure was performed in a cell culture incubator in which the switch of CO_2 was done by a CO_2 controller and target level was reached within 1 min, as determined by the built-in CO_2 sensor. At baseline, the chamber was ventilated with 5% CO_2. CO_2 exposures were achieved with different levels of P_{CO_2} from 20 to 80 mmHg. A stretch of 10 min recordings was

taken. Once the firing rate (FR) of baseline recording was stabilized in three consecutive records, exposures of step-elevated CO$_2$ concentrations were performed subsequently, followed by 30–60 min washout with 5% CO$_2$.

Neuronal response to hypercapnia was studied with at least three levels of P_{CO_2} (50–80 mmHg). In response to hypercapnia, the firing activity of a group of units increased (see below), while others decreased or remained unchanged. To group these units, the peak response was measured first. Then, the sensitivity index C was determined: $\Delta FR/\Delta P_{CO_2}$, where ΔFR is the percentage change in the firing rate and ΔP_{CO_2} is the change in P_{CO_2} (mmHg). The value $C = 0.0067$ corresponds to a 0.67% change in FR per mmHg P_{CO_2}. Our measurements showed that extracellular pH (pH$_o$) decreased from 7.42 to 7.13 with a P_{CO_2} change from 38 to 76 mmHg or 13.2 mmHg P_{CO_2}/pH$_o$ 0.1 unit (Su & Jiang, 2006). Thus, our threshold C value equals an 8.8% change in FR per 0.1 unit pH$_o$. Using this criterion, three groups of units were identified: CO$_2$-stimulated units with $C > 0.0067$, CO$_2$-inhibited units with $C < -0.0067$ and CO$_2$-irresponsive units with $0.0067 > C > -0.0067$. Furthermore, these response patterns had to be reproducible in at least two separate recordings within 24–72 h.

Neurotransmission blockade was performed with antagonist ketanserine tartrate for the 5HT$_{2A}$ receptor, spantide acetate salt for the NK1 receptor, 6cyano-7-nitroquinoxaline-2,3-dione (CNQX) for glutaminergic AMPA/kanate receptor, and pyridoxal phosphate-6-azophenyl-2,4-disulphonic acid tetrasodium salt (PPADS) for the purinergic P2x receptor. Blockade of Kir channels and TASK channels was also performed in the MEA system with Ba^{2+} (BaCl$_2$) and anandamide in water-soluble emulsion (Tocrisolve 100), respectively (Maingret *et al.* 2001; Dwivedi *et al.* 2005; Orie *et al.* 2006). Control experiments were done before and after the chemical treatment with their solvent such as equally diluted Tocrisolve 100 for dissolving anandamide. Spantide was purchased from Sigma Chemicals (St Louis, MO, USA). All other chemicals were purchased from Tocris (Ellisville, MO, USA).

Immunocytochemistry

The cultured cells were processed for fluorescence immunocytochemical staining as previously described (Su & Jiang, 2006), and then examined under a confocal microscope (Zeiss LSM 510). The cells were incubated overnight with primary antibodies: rabbit polyclonal anti-Kir4.1 (1:1000), rabbit polyclonal anti-TASK1 (1:500) (Alomone Laboratory, Israel), goat polyclonal anti-Kir5.1 (1:500; Santa Cruz Biotech, Santa Cruz, CA, USA), and mouse monoclonal anti-MAP2 (1:400; Sigma). Then double or triple labellings were performed by further incubations of cells with the secondary antibodies for 1.5 h: AlexaFluor488-conjugated

donkey anti-rabbit IgG for showing Kir4.1 or TASK1, AlexaFluor594-conjugated donkey anti-goat IgG for showing Kir5.1, AlexaFluor594-conjugated donkey anti-mouse IgG (1:1000; Molecular Probes, Eugene, OR, USA) or AMCA (7-amino-4-methylcoumarin-3-acetic acid) conjugated donkey anti-mouse IgG (1:100; Jackson ImmunoResearch, West Grove, PA, USA) for MAP2. Anti-GFAP (1:400; Sigma) is also used for identifying glial cells in some cultures. In control experiments, the primary antibodies were omitted for Kir5.1 and MAP2/GFAP, or preabsorbed with a threefold excess of the epitope for Kir4.1 (Alomone Laboratory). All of these control experiments showed negative staining.

Whole-cell voltage-clamp recording in *Xenopus* oocytes

Frog oocytes were obtained from *Xenopus laevis*, as previously described (Xu *et al.* 2000; Cui *et al.* 2001). Briefly, female frogs were deeply anaesthetized by bathing in 0.3% 3-aminobenzoic acid ethyl ester, according to IACUC-approved protocols. A few lobs of ovaries were removed and then treated with 0.5 mg ml^{-1} collagenase (Type I; Sigma) in OR2 solution containing (mM): NaCl 82, KCl 2, MgCl$_2$ 1 and Hepes 5 (pH 7.4) for 30 min at room temperature. After several washes with the OR2 solution, oocytes were injected with cDNAs (25–50 ng in 50 nL water) and then incubated at 18°C in ND-96 solution containing (mM): NaCl 96, KCl 2, MgCl$_2$ 1, CaCl$_2$ 1.8, Hepes 5, and sodium pyruvate 2.5, with 100 mg l^{-1} geneticin (pH 7.4).

Rat Kir4.1 (GenBank accession no. X83585) and rat Kir5.1 (GenBank accession no. X83581) cDNAs were generously provided by Dr John Adelman, Oregon Health and Science University. Mouse TASK1 cDNA (GenBank accession no. AAC53367) was a gift from Dr Michel Lazdunski, Institut de Pharmacologie Moleculaire et Cellulaire, France. A tandem Kir4.1–Kir5.1 was constructed, which expresses identical currents as those from coinjection of Kir4.1 and Kir5.1 (Xu *et al.* 2000; Yang *et al.* 2000). The cDNAs were subcloned into a eukaryotic expression vector, pcDNA3.1 (Invitrogen), and used for oocyte expression without cRNA synthesis.

Whole-cell currents were recorded by two-electrode voltage clamp 3 days after cDNA injection using an amplifier (Geneclamp 500; Axon Instruments, Union City, CA, USA) as previously detailed (Xu *et al.* 2000; Cui *et al.* 2001). Ba^{2+} and anandamide sensitivity was tested with extracellular exposure to various concentrations. The concentration–response relationships were expressed using the Hill equation, with IC$_{50}$ determined (Yang & Jiang, 1999; Yang *et al.* 2000). TASK1 channel sensitivity to extracellular pH was studied by using perfusates at various pH levels. Sensitivity of the Kir4.1–Kir5.1 channel to intracellular pH was studied in excised patches, as we have

previously described (Yang & Jiang, 1999; Yang *et al.* 2000). The pH–current relationship was then described with the Hill equation.

Data are presented as means ±s.e.m. Differences between control and treatment groups were examined using ANOVA or Student's two-tailed paired *t* test, and considered to be statistically significant when $P < 0.05$.

Results

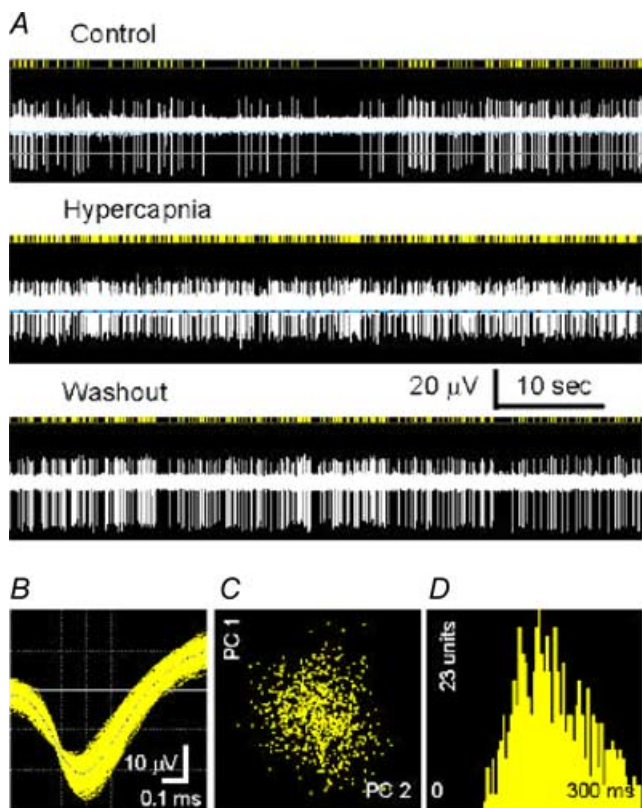### CO$_2$ chemosensitivity of brainstem neurons cultured on MEAs

Neurons were obtained from the medulla oblongata and pons of fetal (day of pregnancy, 17–19) rats and cultured in MEA dishes. The general morphological properties of neurons, in which about 5% were serotonergic neurons, were the same as described in our first paper with the


Figure 1. Recording from a single unit in a microelectrode array (MEA) dish
A, the unit was reversibly stimulated with an exposure to 10% CO$_2$. *B*, the digitized action potentials showed a duration >1 ms. Note that the longer positive wave is not shown. *C*, the principal component analysis (PCA) showed that all spikes detected were clustered in the *X*–*Y* axis system, where the *X* axis is PC2 (i.e. the waveform projection onto the first principal component) and *Y* axis is PC1 (i.e. the waveform projection onto the second principal component). *D*, the interspike interval histogram indicates single-unit recording because of the lack of action potentials in the initial 50 ms.

MEA technique (Su & Jiang, 2006). Glial cells were also present, and consisted of about 30% of the total cell numbers. Extracellular recording was carried out in DMEM at 36°C after 3 weeks of culture (Fig. 1*A*). Single-unit recordings were determined by the methods of principle component analysis (Horn *et al.* 2003) (Fig. 1*B* and *C*) and the absence of action potentials in the initial period of the interspike histogram (Fig. 1*D*). Most spikes showed a negative–positive waveform with a duration >1 ms, suggesting that they were recorded from the soma (Gustafsson & Jankowska, 1976; Jiang & Lipski, 1990; Su & Jiang, 2006). Spikes with small irregular amplitude and narrow duration (0.5–0.7 ms) were occasionally seen. They were considered to be recorded from axons, and they were rejected for further analysis.

In response to hypercapnia, the activity of a group of units increased (Fig. 2*A*), while others decreased or remained unchanged (not shown). Based on the criteria described in Methods, we found 172 CO$_2$-stimulated units, 195 CO$_2$-inhibited units and 448 CO$_2$-irresponsive units out of 815 units from total about 60 fetuses of 6 pregnant rats (see online Supplemental material, Supplemental Fig. 1). The existence of such three groups of cells is consistent with previous findings in brain slices and primary brainstem neuronal culture (Dean *et al.* 1989; Richerson, 1995; Wang *et al.* 1998). In the present study we focused on the CO$_2$-stimulated units only. Some units lost their activity during experimental treatments and were not considered for data analysis.

The CO$_2$-stimulated units showed clear concentration-dependent responses to step changes of P_{CO_2}. When their firing activity was plotted against P_{CO_2}, an almost linear relationship was seen (Fig. 2*A* and *B*). The CO$_2$ response was reversible and reproducible. Evident increase in firing activity of the CO$_2$-stimulated units occurred in 3–5 min when the MEA dish was exposed to elevated CO$_2$. The neuronal firing activity reached a maximum in 7–8 min and was maintained or showed a moderate decline during the rest of the CO$_2$ exposure (Fig. 2*C*). The firing activity resumed to the baseline level in 30–60 min after switching back to 5% CO$_2$. Thus, a 10 min CO$_2$ exposure was used in the studies. Serial concentration–response curves were produced with 2, 3, 4, 5, 7, 9 and 12% CO$_2$. The FR changed linearly around 5% CO$_2$ (P_{CO_2} 38 mmHg) and reached the plateau at ∼9% CO$_2$ (P_{CO_2} 68 mmHg) (Fig. 2*D*).

Some brainstem neurons in MEAs showed high CO$_2$ sensitivity. To determine how high their CO$_2$ sensitivity was, we studied their responses to modest changes of P_{CO_2} in three MEA dishes. Twelve units were identified with *C* values of 0.03 or greater (0.064 ± 0.006, $n = 12$). In response to a 7.6 mmHg P_{CO_2} change, the average FR of these units increased linearly from 1.36 ± 0.29 to 2.63 ± 0.56 Hz ($P < 0.001$, $n = 11$, ANOVA) (Fig. 3). This corresponds to a 16.7% change in FR per 1.0 mmHg

P_{CO_2}, ranging from 4 to 30%, indicating that the CO$_2$ chemosensitivity is well retained in the cultured brainstem neurons.

## Parallel process of P_{CO_2} signals with multiple pH-sensitive K$^+$ channels

Several Kir and TASK channels are modulated by physiological levels of P_{CO_2} and may act as CO$_2$/pH sensors in brainstem neurons (Jiang *et al.* 2005). Selective blockade of the TASK1 channels with 3 μM anandamide (Fig. 4*B*) (Maingret *et al.* 2001; Bai *et al.* 2006; Meuth *et al.* 2006) suppressed neuronal response to severe hypercapnia from 1.35 ± 0.47 to 1.33 ± 0.35 Hz ($P > 0.05$, $n = 38$, paired t test), while neuronal responses to moderate hypercapnia and hypocapnia were barely affected (from 1.35 ± 0.47 to 0.52 ± 0.16 Hz; $P < 0.05$, $n = 38$, paired t test) (Figs 4*A* and *B*, and 5). Blockade of the Kir channels with 30 μM Ba^{2+} (Fig. 4*D*) inhibited the responses of the same neurons to hypocapnia and mild hypercapnia (from 1.50 ± 0.43 to 1.43 ± 0.41 Hz; $P > 0.05$, $n = 38$, paired t test), whereas the FR of these neurons remained to be augmented by 70–80 mmHg P_{CO_2}, though to a less degree (from
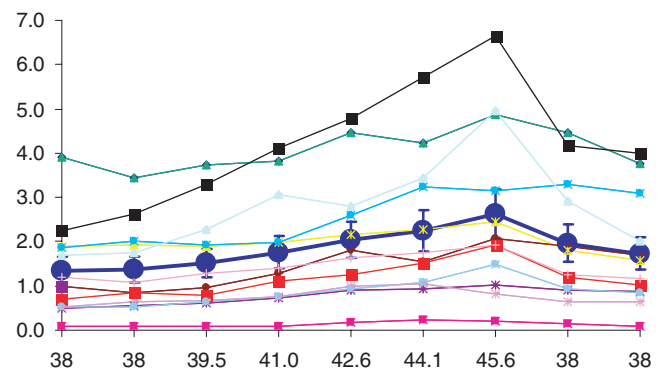
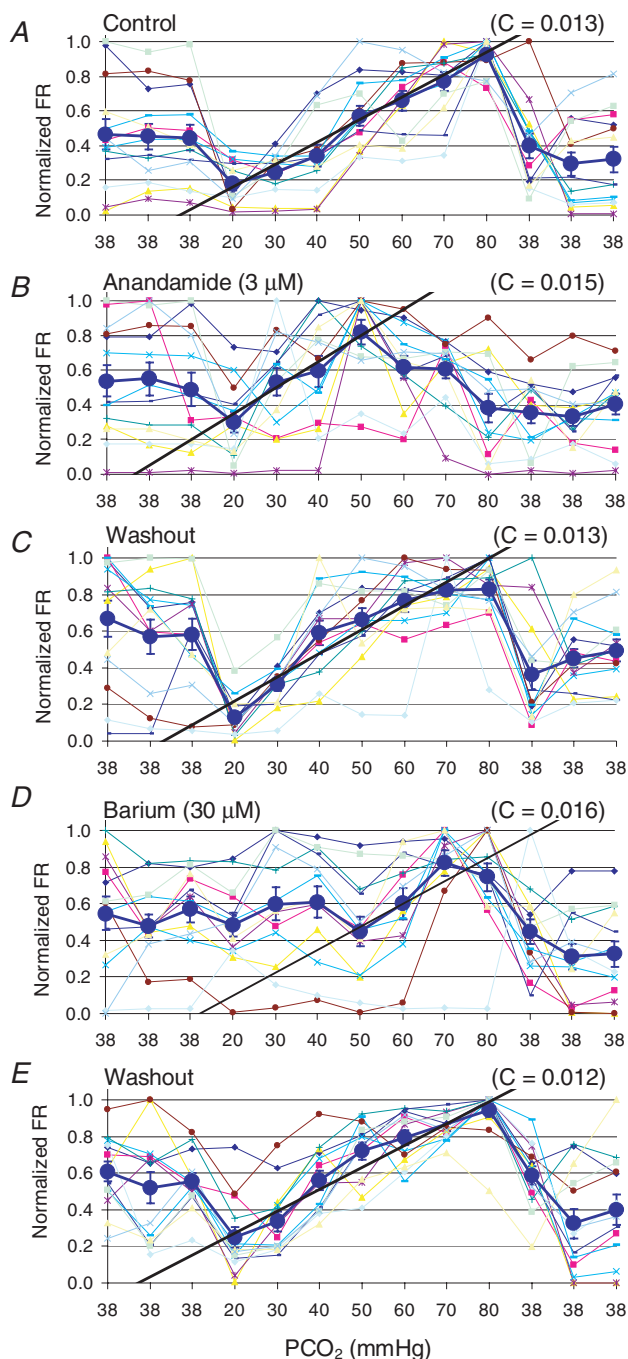**Figure 3. Change in firing activity with modest CO$_2$ changes**
Twelve highly CO$_2$-chemosensitive units were selected from three MEA dishes. These units showed clear changes in their firing activity in response to step changes of CO$_2$ by 1.5 mmHg. The large circles and thick line indicate a linear increase in firing rate from 1.3 to 2.6 Hz ($P < 0.001$, $n = 11$, ANOVA).

1.50 ± 0.43 to 2.18 ± 0.57 Hz; $P < 0.05$, $n = 38$, paired t test) (Figs 4*D* and 5). The CO$_2$ responses were mostly eliminated with high concentrations of Ba^{2+} (up to 500 μM) that block most Kir and TASK channels.
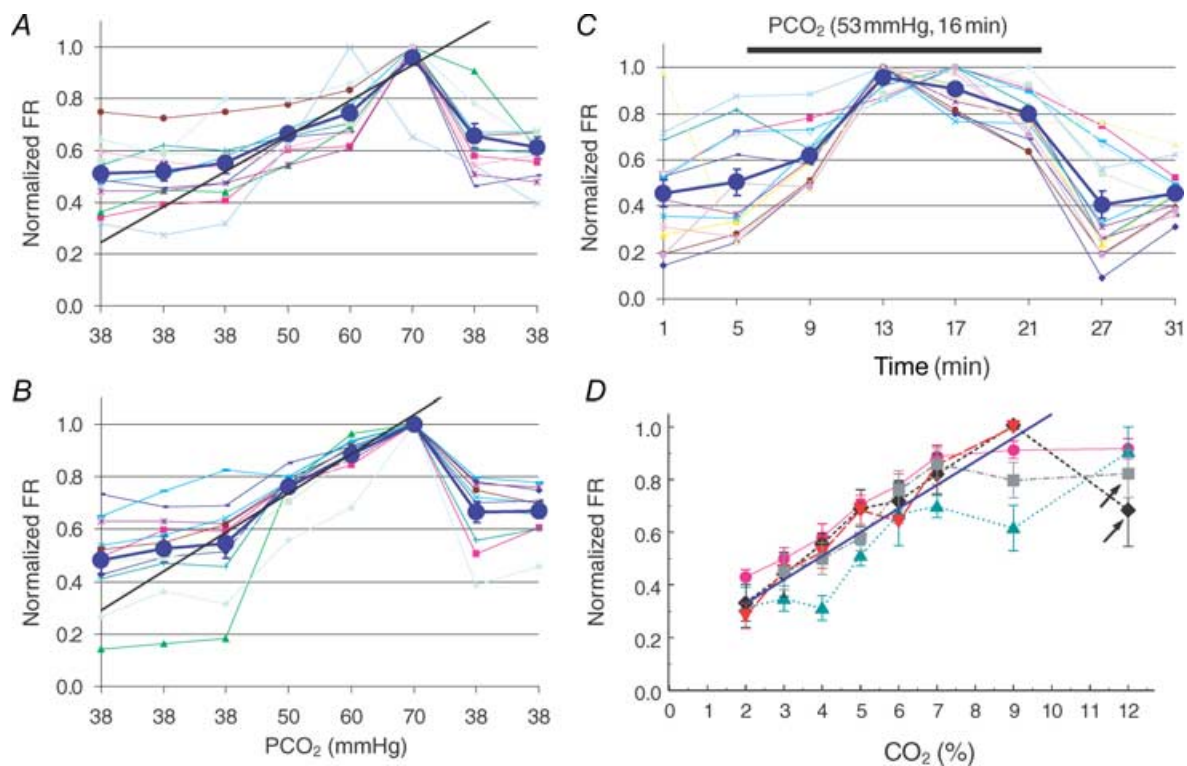
Figure 2. Characterization of CO$_2$-stimulated units
A, in a MEA, CO$_2$ reversibly augmented the firing rate (FR) of 11 units in a concentration-dependent manner (0.55 ± 0.04 to 0.96 ± 0.03; $P < 0.001$, $n = 10$, ANOVA). *B*, the response was reproducible as seen with repetitive exposure in a 24 h interval. Note that each symbol indicates a unit with the average of all units shown as large black circles (0.55 ± 0.05 to 1.00 ± 0.00, $P < 0.001$; $n = 10$, ANOVA). *C*, firing rate of 15 units in another MEA was augmented by 7% CO$_2$, with the maximum response reached in 8 min (0.50 ± 0.06 to 0.80 ± 0.03, $P < 0.001$, $n = 14$, ANOVA). *D*, action potentials of the CO$_2$-stimulated units from four MEA dishes, with one tested twice in 48 h (arrows), were normalized to the peak frequency of each unit during CO$_2$ exposure, averaged in each dish, and displayed as means $\pm$ s.e.m. Note that the straight line indicates a linear change in FR with various levels of P_{CO_2}.

We chose TASK1 and the Kir4.1–Kir5.1 channel, both of which are expressed in the brainstem neurons, to show the pH sensitivity of Kir and TASK channels (Talley *et al.* 2000; Washburn *et al.* 2002; Wu *et al.* 2004; also see Supplemental Fig. 2). These channels were expressed in *Xenopus* oocytes. Using whole-cell voltage clamp, the TASK1 and Kir4.1–Kir5.1 channels were differentiated by their sensitivities to anandamide and Ba^{2+}, respectively (Fig. 6*A–E*). The TASK1 channel showed extracellular pH sensitivity with pK_a 6.65 ($h = 1.9$, $n = 6$; Fig. 6*F*). Since the Kir4.1–Kir5.1 channel is sensitive to intra but not extracellular pH (Xu *et al.* 2000), it was studied in inside-out patches. The Kir4.1–Kir5.1 channel was inhibited by intracellular acidification with pK_a 7.40 ($h = 2.1$, $n = 13$; Fig. 6*F*). Therefore, these results are consistent with the neuronal responses to different levels of P_{CO_2}, further supporting that Kir and TASK channels may work in parallel and cover a wider P_{CO_2} range than they can individually.

Our immunocytochemical assays in cultured brainstem neurons showed that Kir4.1 and Kir5.1 immunoreactivity was colocalized in the same MAP2-positive neurons, which was much denser than in the glial cells. The TASK1 immunostaining was also localized in neurons, although glial cells also had positive stains (Supplemental Fig. 2).

Amplification of CO_2 chemosensitivity by synaptic transmission

The high sensitivity of central chemoreceptors may be attributed to a potential amplification mechanism in the neuronal network. If this is the case, blockade of presynaptic input should reduce neuronal CO_2

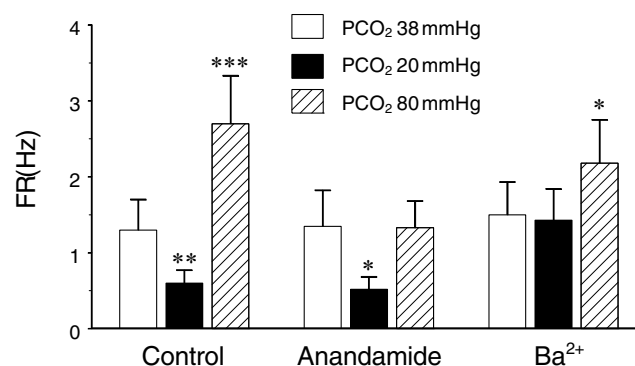**Figure 4. Selective suppression of neuronal response to fractional P_{CO_2}**

A, in a MEA, a nearly linear relationship of the FR (in a 10 min period) with CO_2 concentrations was seen as indicated by the straight line with a value '*C*' showing the change in firing rate divided by the change in P_{CO_2} (0.45 ± 0.08 to 0.93 ± 0.03; $P < 0.001$, $n = 12$, ANOVA). *B*, in the presence of 3 μM anandamide, neuronal sensitivity to P_{CO_2} 70–80 mmHg was abolished (0.49 ± 0.09 to 0.38 ± 0.08; $P > 0.05$, $n = 12$, ANOVA). *D*, in contrast, Ba^{2+} inhibited neuronal responses to P_{CO_2} 20–50 mmHg (0.53 ± 0.06 to 0.45 ± 0.08, $P > 0.05$, $n = 12$). *C* and *E*, evident recovery was seen with washout (0.58 ± 0.08 to 0.83 ± 0.05; $P < 0.001$, $n = 12$, ANOVA in *C*; 0.56 ± 0.04 to 0.94 ± 0.04; $P < 0.001$, $n = 12$, ANOVA in *E*).


Figure 5. Summary of unit responses in the presence of anandamide and Ba^{2+}

In the control, the average FR was significantly inhibited at P_{CO_2} 20 mmHg and augmented at P_{CO_2} 80 mmHg. With 3 μM anandamide, the neuronal response to hypercapnia, but not to hypocapnia, was lost. In the presence of 30 μM Ba^{2+}, the hypocapnic response was suppressed, while neurons remained to be stimulated at P_{CO_2} 80 mmHg. Intra-group differences were examined by comparison to baseline FR (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$, $n = 38$, paired *t* test). Data were obtained from two MEAs.

chemosensitivity. We thereby performed studies to test this hypothesis. Since several groups of serotonergic neurons in the brainstem are CO$_2$ chemosensitive (Wang *et al.* 1998; Severson *et al.* 2003), we used 20 μM ketanserin to block 5HT$_{2A}$ receptors known to play an important role in respiratory control (Richerson, 2004; Tryba *et al.* 2006). Ketanserin markedly diminished neuronal responses to hypercapnia from 4.88 ± 0.84 to 3.07 ± 0.69 Hz ($P < 0.001$, $n = 28$, paired *t* test) (Figs 7 and 8*A*). Amongst 29 CO$_2$-stimulated units in three MEA dishes, only two cells lost their CO$_2$ sensitivity completely. The CO$_2$ chemosensitivity of such a small number of cells was interpreted to be derived solely from presynaptic input. The other 27 units remained to be stimulated by hypercapnia to various degrees. Of these 27 units, 20 showed significant reduction in their CO$_2$ sensitivity, suggesting that the CO$_2$ chemosensitivity of most neurons

is determined by both pre and postsynaptic mechanisms. The CO$_2$ chemosensitivity of the remaining seven units was either retained ($n = 2$) or slightly enhanced ($n = 5$) with ketanserin, suggesting that their CO$_2$ chemosensitivity is independent of serotonergic input or slightly inhibited by serotonergic input.

Another group of candidate CO$_2$ chemoreceptors are glutamatergic neurons, especially those in the retrotrapezoid nucleus (Mulkey *et al.* 2004). We found that neuronal CO$_2$ sensitivity was significantly inhibited from 2.41 ± 0.57 to 1.40 ± 0.44 Hz ($P < 0.001$, $n = 28$, paired *t* test) in the presence of 10 μM CNQX, a non-NMDA receptor antagonist (23 reduced, 2 enhanced, and 4 lost response, out of 29 units in 4 MEAs) (Fig. 8*B*). A similar effect was found with blockade of NK1 receptors (from 1.57 ± 0.40 to 0.85 ± 0.25 Hz; $P < 0.01$, $n = 21$, paired *t* test) in the presence of 100 μM spantide (of 22 units
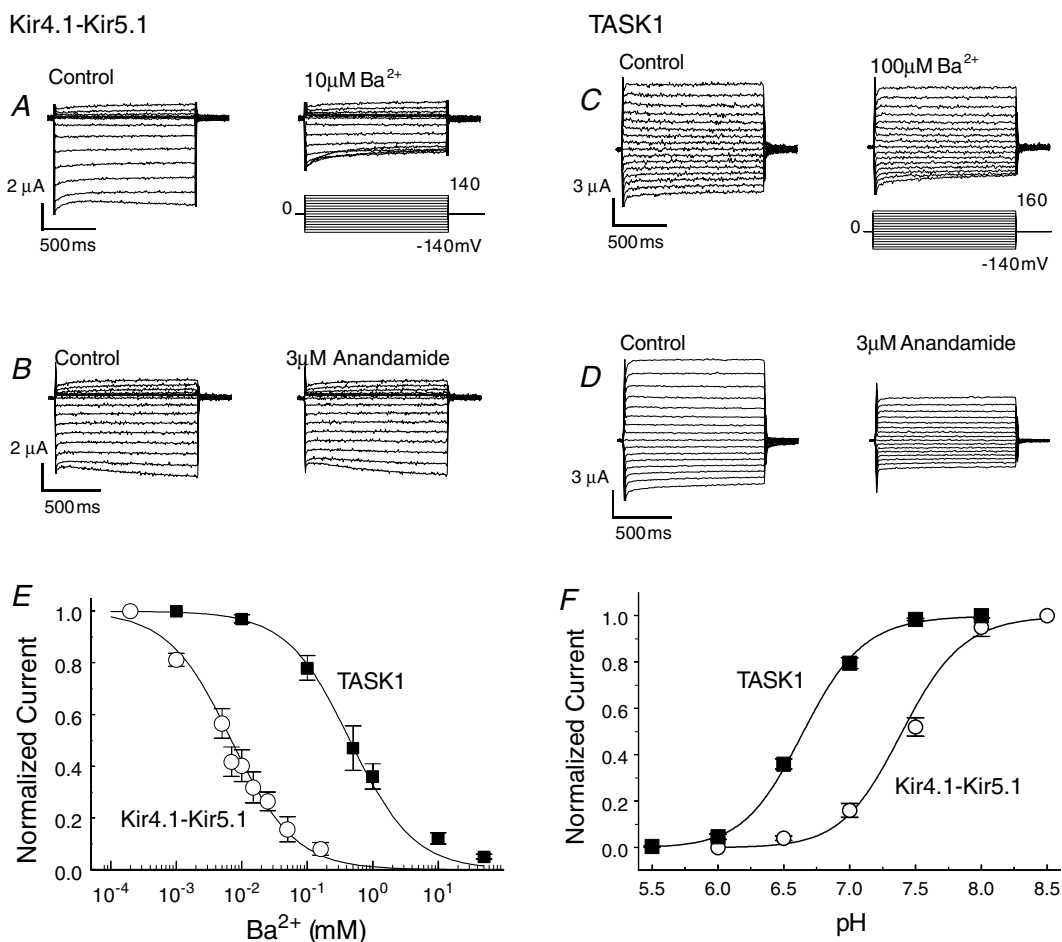
Figure 6. Differential sensitivities of Kir4.1–Kir5.1 channel and TASK1 channel to K$^+$ channel blockers and pH

A–D, whole-cell currents were studied in two-electrode voltage clamp in *Xenopus* oocytes. Ba^{2+} (10 μM) inhibited the Kir4.1–Kir5.1 (*A*) but not the TASK1 channel (*C*). In contrast, the TASK1 channel was more sensitive to anandamide (*D*) than the Kir4.1–Kir5.1 (*B*). *E*, the concentration–response relationship showed different Ba^{2+} sensitivity between the Kir4.1–Kir5.1 (IC$_{50}$ = 7 μM, $n = 4$) and TASK1 (IC$_{50}$ = 450 μM, $n = 4$) channels. *F*, in *Xenopus* oocytes, the Kir4.1–Kir5.1 channel, both of which are expressed in the brainstem (Wu *et al.* 2004), was strongly inhibited at pH 7.0 (pK_a = 7.40, h = 2.1, n = 13), while the TASK-1 channel was inhibited at more acidic pH (pK_a = 6.65, h = 1.9, n = 6).

in 3 MEAs, CO_2 sensitivity was reduced in 17 units, enhanced in 3, and lost in 2; Fig. 8C). In contrast, blockade of purinergic P2x receptors that have been suggested to be involved in CO_2 chemosensitivity (Thomas *et al.* 1999; Gourine *et al.* 2005) with 10 μM PPADS did not show any significant effect (from 2.24 ± 0.79 to 2.66 ± 0.92 Hz; $P > 0.05$, $n = 36$) (Fig. 8D), suggesting that the enhancement of CO_2 chemosensitivity is rather specific to certain synaptic connections.
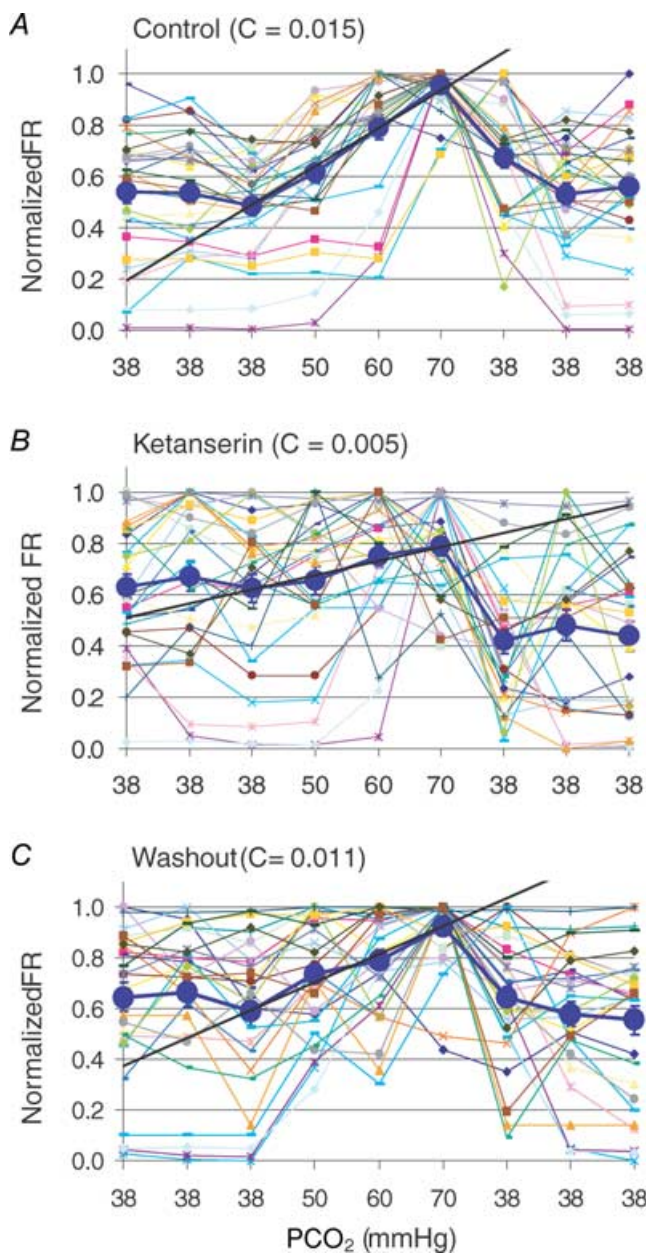
Figure 7. Changes in neuronal CO_2 sensitivity before, during and after ketanserin (20 μM) exposure in two MEA dishes
Neuronal FR was normalized to the peak frequency, and plotted against P_{CO_2} levels. Ketanserin drastically attenuated neuronal response to hypercapnia (0.62 ± 0.06 to 0.79 ± 0.04, $P < 0.05$, $n = 28$, ANOVA).

Discussion

In the present study, we have tested the hypothesis of serial and parallel processing of P_{CO_2} signals using the MEA system. The MEA approach has several advantages
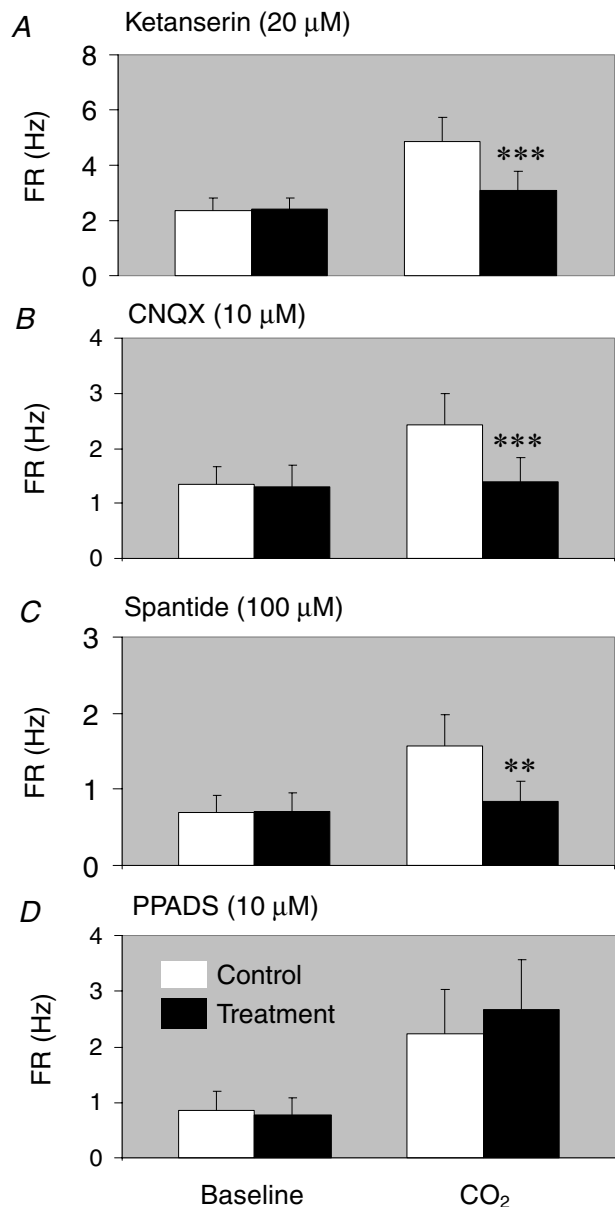
Figure 8. Effects of synaptic blockade on neuronal CO_2 sensitivity
A, when the average FR was compared with and without ketanserin, significant reductions in the FR were seen during CO_2 (70 mmHg) exposure ($P < 0.001$, $n = 28$ from three MEAs, paired t test). B and C, Similar attenuations of the hypercapnic response were observed with CNQX ($P < 0.001$, $n = 28$ from four MEAs, paired t test) and spantide ($P < 0.01$, $n = 21$ from three MEAs, paired t test). D, PPADS, a P2x receptor blocker, did not have significant effect ($P > 0.05$, $n = 36$ from three MEAs, paired t test). Note that the units studied did not show significant differences in their baseline FR with or without the blockers (A–D).

for CO$_2$ chemoreceptor studies. (1) We were able to record repetitively from multiple neurons with each individual unit identified by its firing pattern and CO$_2$ response. (2) All recordings were done in cell culture media at 36°C, in which the neuronal extra and intra-cellular environments were not perturbed. (3) Neuronal CO$_2$ chemosensitivity was studied with several levels of CO$_2$ instead of a single dosage, allowing an estimation of the concentration–response relationship and the response pattern of individual neurons. (4) Action potentials were sorted with the method of principal component analysis before data analysis (Horn *et al.* 2003), so that the identification and isolation of single units was greatly improved over the traditional window discriminators. (5) Our results suggest that the neuronal inherent membrane properties and synaptic interaction necessary for CO$_2$ chemosensitivity seem to be retained in such a reduced neuronal network. Although the neurons lost their surrounding tissues, they were more accessible for treatments with CO$_2$ and other pharmacological agents. With the MEA system, we have shown evidence for neuronal processing of the CO$_2$ signals in which the P_{CO_2} spectrum seems to be covered by multiple sensing molecules and the sensitivity is enhanced with the CO$_2$ chemosensitivity of both pre and postsynaptic neurons.

Our results have shown that ∼20% of cultured neurons in MEAs were stimulated by hypercapnia. The increase of the FR by elevated CO$_2$ was reversible and reproducible. With step elevations in P_{CO_2} levels, we have found that neurons in the MEA showed a linear response to the range of P_{CO_2} from 20 to 80 mmHg, which is consistent with previous *in vivo* studies with respect to CO$_2$ chemo-sensitivity (Nattie, 1999). Some neurons showed high CO$_2$ sensitivity by changing their FR robustly in response to modest hypercapnia. Their average response is 16.7% per 1.0 mmHg P_{CO_2}, corresponding to ∼220% increase in FR per 0.1 pH$_o$ units, which falls in the upper range of CO$_2$ sensitivity among all brainstem neurons (Putnam *et al.* 2004). These results suggest that cultured brainstem neurons still retain high CO$_2$ chemosensitivity in a broad sensing spectrum as seen in other brainstem preparations (Putnam *et al.* 2004).

Previous studies have demonstrated the presence of CO$_2$ chemosensitivity in both pre and postsynaptic neurons (Richerson, 1995; Kawai *et al.* 1996, 2006; Stunden *et al.* 2001; Takakura *et al.* 2006), which we inferred may allow an amplification of P_{CO_2} signals detected by each cell. Consistent with this scenario, our results indicate that CO$_2$ chemosensitivity is significantly reduced when 5-HT$_{2A}$, NK1 or non-NMDA receptors are blocked, all of which are receptors for neurotransmitters that play an important role in the modulation of central respiratory activity and CO$_2$ chemosensitivity (Feldman *et al.* 2003; Richerson, 2004; Guyenet *et al.* 2005*b*). Blockade of the P2x receptor, however, did not produce evident attenuation of the

CO$_2$ chemosensitivity, consistent with two recent studies showing lack of effect on the CO$_2$ chemosensitivity by P2x receptor blockade (Lorier *et al.* 2004; Mulkey *et al.* 2006). It is possible that the brainstem neurons that are intrinsically CO$_2$ chemosensitive may not receive direct purinergic synaptic input as suggested by Mulkey *et al.* (2006). It is worth emphasizing that the serial process should not be limited to two brainstem neurons. Instead, the signal amplification is likely to involve multiple interneurons, peripheral afferents and perhaps certain motoneurons as well, as some of them are pH sensitive as well (Harada *et al.* 1985; Talley *et al.* 2000; Takakura *et al.* 2006).

Several ion channels and receptors are modulated by physiological levels of P_{CO_2}/pH and may act as CO$_2$/pH sensors in brainstem neurons (Dean *et al.* 1997; Thomas *et al.* 1999; Talley *et al.* 2000; Richerson, 2001; Putnam *et al.* 2004; Jiang *et al.* 2005). Our results suggest that some of these CO$_2$/pH sensing molecules such as Kir channels and TASK channels play a part in detecting different levels of P_{CO_2} in the same cells. While high levels of hypercapnia are likely to affect the TASK channels, the Kir channels appear to act in mild hypercapnia and hypocapnia. These observations are well supported by our whole-cell voltage-clamp studies of TASK1 and Kir4.1–Kir5.1 channels in the *Xenopus* oocyte expression system, showing that the pH sensing ranges of these channels are consistent with the neuronal responses. Besides the Kir and TASK channels, our experiments do not exclude the possibility that other sensing molecules may play a similar role in the CO$_2$ chemosensitivity. The wide distribution of multiple CO$_2$/pH sensing molecules may thus allow brainstem neurons to cover the P_{CO_2} spectrum using a similar parallel process.

In conclusion, although a good number of brainstem neurons are inherently CO$_2$ chemosensitive, their CO$_2$ sensitivity does not seem adequate to produce a 20–30% change in systemic respiratory response to 1 mmHg P_{CO_2}. Our results suggest that the physiological range of P_{CO_2} seems to be covered by multiple CO$_2$/pH sensing molecules that respond to different levels of P_{CO_2}, and the CO$_2$ chemosensitivity is strongly augmented with synaptic transmission involving 5-HT$_{2A}$, NK1 and non-NMDA receptors. Therefore, the spectrum problem appears to be solved by a parallel process of the P_{CO_2} signals by multiple sensing molecules, and the sensitivity problem is eased with cellular mechanisms involving synaptic amplifications by CO$_2$-chemosensitive neurons arranged in serial in cultured brainstem neurons.

References

Bai XL, Lacey HA, Greenwood SL, Baker PN, Turner MA, Sibley CP & Fyfe GK (2006). TASK channel expression in human placenta and cytotrophoblast cells. *J Soc Gynecol Invest* **13**, 30–39.

Bradley SR, Pieribone VA, Wang WG, Severson CA, Jacobs RA & Richerson GB (2002). Chemosensitive serotonergic neurons are closely associated with large medullary arteries. *Nat Neurosci* **5**, 401–402.

Cui N, Giwa LR, Xu H, Rojas A, Abdulkadir L & Jiang C (2001). Modulation of the heteromeric Kir4.1-Kir5.1 channels by P_{CO_2} at physiological levels. *Am J Physiol Cell Physiol* **189**, C229–C236.

Dean JB, Huang RQ, Erlichman JS, Southard TL & Hellard DT (1997). Cell-cell coupling occurs in dorsal medullary neurons after minimizing anatomical-coupling artifacts. *Neuroscience* **80**, 21–40.

Dean JB, Lawing WL & Millhorn DE (1989). CO_2 decreases membrane conductance and depolarizes neurons in the nucleus tractus solitarii. *Exp Brain Res* **76**, 656–661.

Dwivedi R, Saha S, Chowienczyk PJ & Ritter JM (2005). Block of inward rectifying K^+ channels (K-IR) inhibits bradykinin-induced vasodilatation in human forearm resistance vasculature. *Arterioscler Thromb Vasc Biol* **25**, E7–E9.

Feldman JL, Mitchell GS & Nattie EE (2003). Breathing: rhythmicity, plasticity, chemosensitivity. *Annu Rev Neurosci* **26**, 239–266.

Filosa JA & Putnam RW (2003). Multiple targets of chemosensitive signaling in locus coeruleus neurons: role of K^+ and Ca^{2+} channels. *Am J Physiol Cell Physiol* **284**, C145–C155.

Gourine AV, Llaudet E, Dale N & Spyer KM (2005). ATP is a mediator of chemosensory transduction in the central nervous system. *Nature* **436**, 108–111.

Gustafsson B & Jankowska E (1976). Direct and indirect activation of nerve cells by electrical pulses applied extracellularly. *J Physiol* **258**, 33–61.

Guyenet PG, Mulkey DK, Stornetta RL & Bayliss DA (2005*a*). Regulation of ventral surface chemoreceptors by the central respiratory pattern generator. *J Neurosci* **25**, 8938–8947.

Guyenet PG, Stornetta RL, Bayliss DA & Mulkey DK (2005*b*). Retrotrapezoid nucleus: a litmus test for the identification of central chemoreceptors. *Exp Physiol* **90**, 247–253.

Harada Y, Wang YZ & Kuno M (1985). Central chemosensitivity to H^+ and CO_2 in the rat respiratory center in vitro. *Brain Res* **333**, 336–339.

Horn CC & Friedman MI (2003). Detection of single unit activity from the rat vagus using cluster analysis of principal components. *J Neurosci Methods* **122**, 141–147.

Jiang C & Lipski J (1990). Extensive monosynaptic inhibition of ventral respiratory group neurons by augmenting neurons in the Botzinger complex in the cat. *Exp Brain Res* **81**, 639–648.

Jiang C, Rojas A, Wang RP & Wang XR (2005). CO_2 central chemosensitivity: why are there so many sensing molecules? *Respir Physiol Neurobiol* **145**, 115–126.

Kawai A, Ballantyne D, Muckenhoff K & Scheid P (1996). Chemosensitive medullary neurones in the brainstem–spinal cord preparation of the neonatal rat. *J Physiol* **492**, 277–292.

Kawai A, Onimaru H & Homma I (2006). Mechanisms of CO_2/H^+ chemoreception by respiratory rhythm generator neurons in the medulla from newborn rats *in vitro*. *J Physiol* **572**, 525–537.

Lorier AR, Peebles K, Brosenitsch T, Robinson DM, Housley GD & Funk GD (2004). P2 receptors modulate respiratory rhythm but do not contribute to central CO_2 sensitivity in vitro. *Respir Physiol Neurobiol* **142**, 27–42.

Maingret F, Patel AJ, Lazdunski M & Honore E (2001). The endocannabinoid anandamide is a direct and selective blocker of the background K^+ channel TASK-1. *EMBO J* **20**, 47–54.

Meuth SG, Aller MI, Munsch T, Schuhmacher T, Seidenbecher T, Meuth P, Kleinschnitz C, Pape HC, Wiendl H, Wisden W & Budde T (2006). The contribution of TWIK-related acid-sensitive K^+-containing channels to the function of dorsal lateral geniculate thalamocortical relay neurons. *Mol Pharmacol* **69**, 1468–1476.

Mulkey DK, Mistry AM, Guyenet PG & Bayliss DA (2006). Purinergic P2 receptors modulate excitability but do not mediate pH sensitivity of RTN respiratory chemoreceptors. *J Neurosci* **26**, 7230–7233.

Mulkey DK, Stornetta RL, Weston MC, Simmons JR, Parker A, Bayliss DA & Guyenet PG (2004). Respiratory control by ventral surface chemoreceptor neurons in rats. *Nat Neurosci* **7**, 1360–1369.

Nattie E (1999). CO_2, brainstem chemoreceptors and breathing. *Prog Neurobiol* **59**, 299–331.

Okada Y, Chen Z, Jiang W, Kuwana S & Eldridge FL (2002). Anatomical arrangement of hypercapnia-activated cells in the superficial ventral medulla of rats. *J Appl Physiol* **93**, 427–439.

Onimaru H, Arata A & Homma I (1989). Firing properties of respiratory rhythm generating neurons in the absence of synaptic transmission in rat medulla in vitro. *Exp Brain Res* **76**, 530–536.

Orie NN, Fry CH & Clapp LH (2006). Evidence that inward rectifier K^+ channels mediate relaxation by the PGI_2 receptor agonist cicaprost via a cyclic AMP-independent mechanism. *Cardiovasc Res* **69**, 107–115.

Oyamada Y, Andrzejewski M, Muckenhoff K, Scheid P & Ballantyne D (1999). Locus coeruleus neurones in vitro: pH-sensitive oscillations of membrane potential in an electrically coupled network. *Respir Physiol* **118**, 131–147.

Oyamada Y, Ballantyne D, Muckenhoff K & Scheid P (1998). Respiration-modulated membrane potential and chemosensitivity of locus coeruleus neurones in the *in vitro* brainstem–spinal cord of the neonatal rat. *J Physiol* **513**, 381–398.

Pineda J & Aghajanian GK (1997). Carbon dioxide regulates the tonic activity of locus coeruleus neurons by modulating a proton and polyamine-sensitive inward rectifier potassium current. *Neuroscience* **77**, 723–743.

Potter SM & DeMarse TB (2001). A new approach to neural cell culture for long-term studies. *J Neurosci Methods* **110**, 17–24.

Putnam RW, Filosa JA & Ritucci NA (2004). Cellular mechanisms involved in CO_2 and acid signaling in chemosensitive neurons. *Am J Physiol Cell Physiol* **287**, C1493–C1526.

Richerson GB (1995). Response to CO_2 of neurons in the rostral ventral medulla *in vitro*. *J Neurophysiol* **73**, 933–944.

Richerson GB (2004). Serotonergic neurons as carbon dioxide sensors that maintain pH homeostasis. *Nat Rev Neurosci* **5**, 449–461.

Richerson GB, Wang WG, Tiwari J & Bradley SR (2001). Chemo sensitivity of serotonergic neurons in the rostral ventral medulla. *Respir Physiol* **129**, 175–189.

Ritucci NA, Erlichman JS, Leiter JC & Putnam RW (2005). Response of membrane potential and intracellular pH to hypercapnia in neurons and astrocytes from rat retrotrapezoid nucleus. *Am J Physiol Regul Integr Comp Physiol* **289**, R851–R861.

Severson CA, Wang WG, Pieribone VA, Dohle CI & Richerson GB (2003). Midbrain serotonergic neurons are central pH chemoreceptors. *Nat Neurosci* **6**, 1139–1140.

Stunden CE, Filosa JA, Garcia AJ, Dean JB & Putnam RW (2001). Development of *in vivo* ventilatory and single chemosensitive neuron responses to hypercapnia in rats. *Respir Physiol* **127**, 135–155.

Su J & Jiang C (2006). Multicellular recordings of cultured brainstem neurons in microelectrode arrays. *Cell Tissue Res* **326**, 25–33.

Takakura ACT, Moreira TS, Colombari E, West GH, Stornetta RL & Guyenet PG (2006). Peripheral chemoreceptor inputs to retrotrapezoid nucleus (RTN) CO$_2$-sensitive neurons in rats. *J Physiol* **572**, 503–523.

Talley EM, Lei QB, Sirois JE & Bayliss DA (2000). TASK1, a two-pore domain K$^+$ channel, is modulated by multiple neurotransmitters in motoneurons. *Neuron* **25**, 399–410.

Thomas T, Ralevic V, Gadd CA & Spyer KM (1999). Central CO$_2$ chemoreception: a mechanism involving P2 purinoceptors localized in the ventrolateral medulla of the anaesthetized rat. *J Physiol* **517**, 899–905.

Tryba AK, Pena F & Ramirez JM (2006). Gasping activity *in vitro*: a rhythm dependent on 5-HT$_{2A}$ receptors. *J Neurosci* **26**, 2623–2634.

Wang WG, Pizzonia JH & Richerson GB (1998). Chemosensitivity of rat medullary raphe neurones in primary tissue culture. *J Physiol* **511**, 433–450.

Washburn CP, Sirois JE, Talley EM, Guyenet PG & Bayliss DA (2002). Serotonergic raphe neurons express TASK channel transcripts and a TASK-like pH and halothane-sensitive K$^+$ conductance. *J Neurosci* **22**, 1256–1265.

Wu J, Xu H, Shen W & Jiang C (2004). Expression and coexpression of CO$_2$-sensitive Kir channels in brainstem neurons of rats. *J Membr Biol* **197**, 179–191.

Xu H, Cui N, Yang Z, Qu Z & Jiang C (2000). Modulation of kir4.1 and kir5.1 by hypercapnia and intracellular acidosis. *J Physiol* **524**, 725–735.

Yang Z & Jiang C (1999). Opposite effects of pH on open-state probability and single channel conductance of kir4.1 channels. *J Physiol* **520**, 921–927.

Yang Z, Xu H, Cui N, Qu Z, Chanchevalap S, Shen W & Jiang C (2000). Biophysical and molecular mechanisms underlying the modulation of heteromeric Kir4.1–Kir5.1 channels by CO$_2$ and pH. *J Gen Physiol* **116**, 33–45.

Acknowledgements

Supplemental material

The online version of this paper can be accessed at:
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and contains supplemental material consisting of two figures:

Supplemental Figure 1. Summary of sensitive index of CO$_2$-stimulated, CO$_2$-unresponsive and CO$_2$-inhibited neuron

Supplemental Figure 2. Immunocytochemical staining of Kir4.1, Kir5.1 and TASK1 channels in cultured brainstem cells

This material can also be found as part of the full-text HTML version available from http://www.blackwell-synergy.com